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Applicant:	Richard S. Blumberg
Serial No.:	09/884,196
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For:	T CELL INHIBITORY RECEPTOR COMPOSITIONS AND USES THEREOF
Examiner:	VanderVegt, F. Pierre
Art Unit:	1644

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF RICHARD S. BLUMBERG UNDER 37 C.F.R. § 1.132

Sir:

I, Richard S. Blumberg, declare that:

1. I am an inventor of the above-identified patent application. I make this declaration in support of that application and in response to the Office Action (paper no. 10) dated July 29, 2003.

2. The purpose of this declaration is to describe results of experiments conducted using the *in vivo* methods disclosed in the above-identified application. The experiments described herein were conducted by me or were conducted under my direct supervision and control.

3. In this declaration, I use the more commonly accepted term for biliary glycoprotein, "carcinoembryonic antigen cell adhesion molecule 1" (CEACAM1). CEACAM1 is an immune receptor tyrosine-based inhibitory motif-containing cell surface molecule that has been proposed to negatively regulate T cell function.

4. To show the data obtained from the practice of the claimed invention, I submit herewith as Exhibits several figures that are included in a manuscript submitted for publication. The figures are labeled Figs. 1, 2 and 5, and are referred to as such in this declaration. Also included as exhibits are the figure legends for the aforementioned figures, which describe the particular results of the figures.

5. To demonstrate the effect of a biliary glycoprotein binding agent *in vivo*, as described in the above-identified application, two T cell-dependent, hapten-mediated colitis mouse models were used: 2,4,6,-trinitrobenzene sulfonic acid (TNBS) colitis and 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone) colitis. In these models, colitis is induced by painting the mouse skin with the respective haptenating agent on day 1 followed by intrarectal administration of the agent on day 8. The result of this treatment is that mice develop colitis as manifested in weight loss, shortening of the colon and thickening of the colon. Specifics of the models have been described in Nieuwenhuis et al., 2002, Proc Natl Acad Sci U S A 99:16951-16956, and Neurath, et al., 2002, J Exp Med 195:1129-1143.

6. *In vivo* treatment with CC1 monoclonal antibody (mAb) and CEACAM1-Fc fusion protein was performed as follows. The anti-CEACAM1[a] mAb, CC1, was purified from a hybridoma derived by fusion of SP20 cells with spleen cells from SJL/J mice immunized with purified BALB/c intestinal brush border membranes. Control mouse IgG1 mAb directed against irrelevant antigen (the B subunit of cholera toxin) were purified by standard methods. Either 1 mg of the CC1 or control mouse IgG1 mAb was administered 24 h before skin-painting and/or rectal challenge with the haptenating reagent. C57BL/6 mice with oxazolone colitis were injected with N-CEACAM1-Fc fusion protein (200 μg/dosage; Gallagher, 1997. J Virol 71:3129-3137), every other day starting one day prior to skin painting and ending one day before sacrificing the mice.

7. Grading of histologic changes was performed as follows. Colonic tissues were removed on day 12 and embedded in paraffin for staining with hematoxylin and eosin. The degree of inflammation on microscopic cross-sections of the colon was graded semiquantitatively. For the histopathologic grading of TNBS-induced colitis, the severity of colitis was assessed based on

two criteria, inflammation and injury. Inflammation was graded from 0 to 4 (0; none, 1; low, 2; moderate, 3; high, 4; severe with transmural leukocyte infiltration, loss of goblet cells) and severity of injury was graded from 0 to 3 (0; none, 1; occasional epithelial lesion, 2; clear-cut ulceration, 3; extensive ulceration). For histopathologic grading of oxazolone-induced colitis, five criteria (hyper-vascularization, presence of mononuclear cells, epithelial hyperplasia, epithelial injury, and presence of granulocytes) were scored from 0 to 3 in each with an additive score between 0 (no colitis) and 15 (maximal colitis activity). Grading was performed in a blinded fashion by the same pathologist.

8. Cytokine assays were performed as follows. Lamina propria mononuclear cells ($5x10^5$ cells/well) isolated on day 12 were stimulated with plate-bound hamster anti-mouse CD3ε mAb (5 μg/ml; BD PharMingen) and soluble hamster anti-mouse CD28 mAb (2 μg/ml; BD PharMingen). Culture supernatants were harvested at 48 h after the cell culture and concentrations of IFN-γ, IL-2, IL-4, and tumor necrosis factor (TNF)-α were measured with OptEIA ELISA sets (BD PharMingen).

9. To test whether CEACAM1[a] is involved in T cell-mediated colonic inflammation at the time of T cell priming by DC or at the time of the effector response by intestinal T cells, the effects of CC1 mAb were assessed at the time of either T cell priming (prior to skin painting) and/or the effector phase (prior to rectal challenge) in the TNBS colitis model. The results are shown in Figure 1. Animals that received TNBS in association with either a control mAb (open squares) or the CC1 mAb prior to skin painting (closed circles) experienced severe weight loss. In contrast, mice that received the CC1 mAb either prior to rectal challenge (open circles) or both skin painting and rectal challenge (twice, closed squares) experienced less weight loss (Fig. 1a). This was directly reflected in the levels of macroscopic injury observed in that mice treated with the CC1 mAb either prior to rectal challenge or twice did not exhibit significant shortening and thickening of the colon (Fig. 1b). Consistent with these macroscopic changes, the control mAb-treated group and groups receiving the CC1 mAb only prior to skin painting exhibited marked, transmural infiltration with inflammatory cells and injury with ulceration (Fig. 1c, panels A and C, respectively). In contrast, mice treated with the CC1 mAb either prior to rectal challenge or twice exhibited less severe histologic features of colitis (Fig. 1c, panels B and D,

respectively). When quantified by a histologic scoring system for evidence of inflammation and injury, these histologic challenges were highly significant (Fig. 1d).

10. To determine whether this mAb CC1-mediated protection from colitis was associated with alterations in cytokine production, Th1 (IFN-γ) and Th2 (IL-4)-associated cytokine production by lamina propria lymphocytes (LPLs) was assessed in the various treatment groups. These studies revealed a significant reduction in IFN-γ production by LPL from mice that received the CC1 mAb either prior to rectal challenge or twice, but not under the other conditions examined (Fig. 1e). Interestingly, no effects were observed on Th2 cytokine production as defined by assessment of IL-4. A similar inhibition of IFN-γ but not IL-4 as a consequence of CC1 mAb treatment was observed with splenocytes stimulated with anti-CD3 plus anti-CD28 mAbs (data not shown). Moreover, the quantities of $CD4^+$ T cells within the lamina propria did not differ between CC1-treated and untreated animals indicating that the effects observed were not simply due to decreased T cells in this compartment (data not shown). These results indicate that suppression of TNBS colitis by administration of the CC1 mAb is associated with a specific reduction in production of IFN-γ, but not IL-4, which is consistent with the role of IFN-γ in this model of colitis (Neurath, et al., 1995. J Exp Med 182:1281-1290).

11. To determine whether these effects of CEACAM1 on the development of T cell mediated immunopathology in the colon could be extended to a Th2-associated colitis, the effect of the CC1 mAb was analyzed in the oxazolone colitis model, which has been previously shown to be primarily Th2-mediated in the SJL strain of mice (Boirivant, et al. 1998. J Exp Med 188:1929-1939). The same general effects of CC1 mAb on TNBS colitis on the body weight were also evident in oxazolone colitis (Fig. 2a). Specifically, mice treated with the CC1 mAb either prior to rectal challenge (open circles) or twice (closed squares) and, to a lesser extent, prior to skin painting (closed circles) exhibited less weight loss than mice treated with the control mAb (open squares). An assessment for macroscopic evidence of colon shortening confirmed these clinical findings (Fig. 2b). Histologic analysis also revealed that colon tissues from mice treated with the CC1 mAb either prior to rectal challenge or twice exhibited significantly less severe histologic evidence of colitis (Fig. 2c, panel B and D). In contrast, the control mAb-treated mice or mice treated with CC1 mAb prior to skin sensitization developed severe colitis (Fig. 2c, panel A and

C). When analyzed quantitatively with a scoring system previously applied to this model, these differences were highly significant (Fig. 2d).

12. The oxazolone colitis model was originally reported to be a predominantly Th2-mediated model in SJL mice (Boirivant, et al. 1998), although in other studies it has been shown that in fact oxazolone colitis is a mixed Th1 and Th2 colitis in the C57BL/6J background as opposed to the SJL background. These results were therefore interesting in that they would have predicted that CEACAM1 was regulating Th2 pathways. Quite surprisingly, however, when the cytokines produced by LPLs after anti-CD3 plus anti-CD28 stimulation were analyzed in this model, IFN-γ, but not the Th2 cytokine (IL-4), was significantly decreased in C57BL/6 mice that received the CC1 mAb either prior to rectal challenge or twice (Fig. 2e). Other regulatory cytokines [e.g., transforming growth factor (TGF)-β, IL-10] and Th1/Th2 cytokines [e.g., tumor necrosis factor (TNF)-α, IL-2, IL-5, IL-10, IL-13)] were not affected by CC1 mAb treatment in both the TNBS and oxazolone colitis (data not shown). Thus, as observed in the TNBS colitis model, ligation of CEACAM1^a by the CC1 mAb at the time of the effector phase of oxazolone-induced colitis caused a decrease in colonic immunopathology that was associated with specific inhibition of the Th1 cytokine, IFN-γ, but not either Th2 or regulatory cytokines.

13. In addition, the effects of a CEACAM1^a-Fc fusion protein (sMHVR-Ig) encoding the extracellular portion of the mCEACAM1^a-4L isoform that has been shown to homophilically ligate the CEACAM1^a molecule *in vitro* (Gallagher, 1997) were examined *in vivo*. Mice were treated with either the CEACAM1^a-Fc fusion protein or a control isotype matched Fc-fragment at a dose of 200 μg administered every other day for 7 doses beginning at the time of skin sensitization in the oxazolone colitis model (Heller et al., 2002, Immunity, 17:629–638). Macroscopic assessment revealed that shortening of the colon was less severe in the mice treated with CEACAM1^a-Fc compared to the mice administered the control-Fc fragment (Fig. 5a). Similarly, histologic evidence of colitis was less pronounced in the CEACAM1^a-Fc-treated group compared to the control-Fc-treated group (Fig. 5b and c). These pathologic finding were associated with a significant decrease in IFN-γ production by LPLs from mice treated with the CEACAM1^a-Fc fusion protein (Fig. 5d). The fact that the decrease in immunopathologic injury observed occurred in association with suppression of IFN-γ production supported that these were causally related.

14. The observation that a CEACAM1^a-Fc fusion caused a reduction in IFN-γ production and associated immunopathology suggests that homophilic ligation of CEACAM1 on antigen-activated T cells leads to induction of an inhibitory pathway in T cells that is specifically linked to Th1 pathways. These studies thus suggest that CEACAM1^a on activated T cells is the target of the CC1 mAb and that the CC1 mAb induced downregulation of IFN-γ production by T cells is likely to play an indispensable role in the suppression of the colitis observed. Moreover, the fact that production of Th2 cytokines was not affected by the CC1 mAb in the oxazolone colitis model which is a mixed Th1/Th2 model in the C57BL/6J background further suggests that CEACAM1^a is directly and specifically linked to regulation of Th1 but not Th2 pathways in this model. Taken together with the observation that a chimeric CEACAM1-Fc fusion, which is known to bind homophilically to CEACAM1^a, could also reduce immunopathology and Th1 cytokine production *in vivo*, our results suggest that homophilic (CEACAM1^a) or heterophilic (antibody) ligation of CEACAM1^a can directly activate an inhibitory pathway on T cells that is linked to Th1 cytokine production.

15. In summary, the experimental results show that CEACAM1^a is associated with specific regulation of Th1 pathways *in vivo* as defined by an analysis of two T cell dependent, hapten-mediated colitis models. Based upon an analysis of the effects of anti-CEACAM1^a antibodies and a CEACAM1^a -Fc chimeric protein *in vivo*, the regulation of Th1 pathways by CEACAM1^a is likely due to either homophilic or heterophilic ligation of the CEACAM1^a N-domain on activated T cells. These results directly demonstrate an *in vivo* role for CEACAM1 as a functionally important activation-induced regulatory molecule on T cells and show that the major functional effect of CEACAM1 on T cells *in vivo* is inhibitory. Moreover, these studies point toward an important role for CEACAM1 in regulating Th1-mediated inflammation such as that associated with inflammatory bowel disease.

16. From the results described herein, one of ordinary skill in the art would recognize that specific suppression of killer T cells in a mixed population of cells and *in vivo* can be performed in accordance with the disclosure of the application.

I, Richard S. Blumberg, declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this document and any patent which may issue from the above-identified patent application.

Date: 11/26/03

Richard S. Blumberg

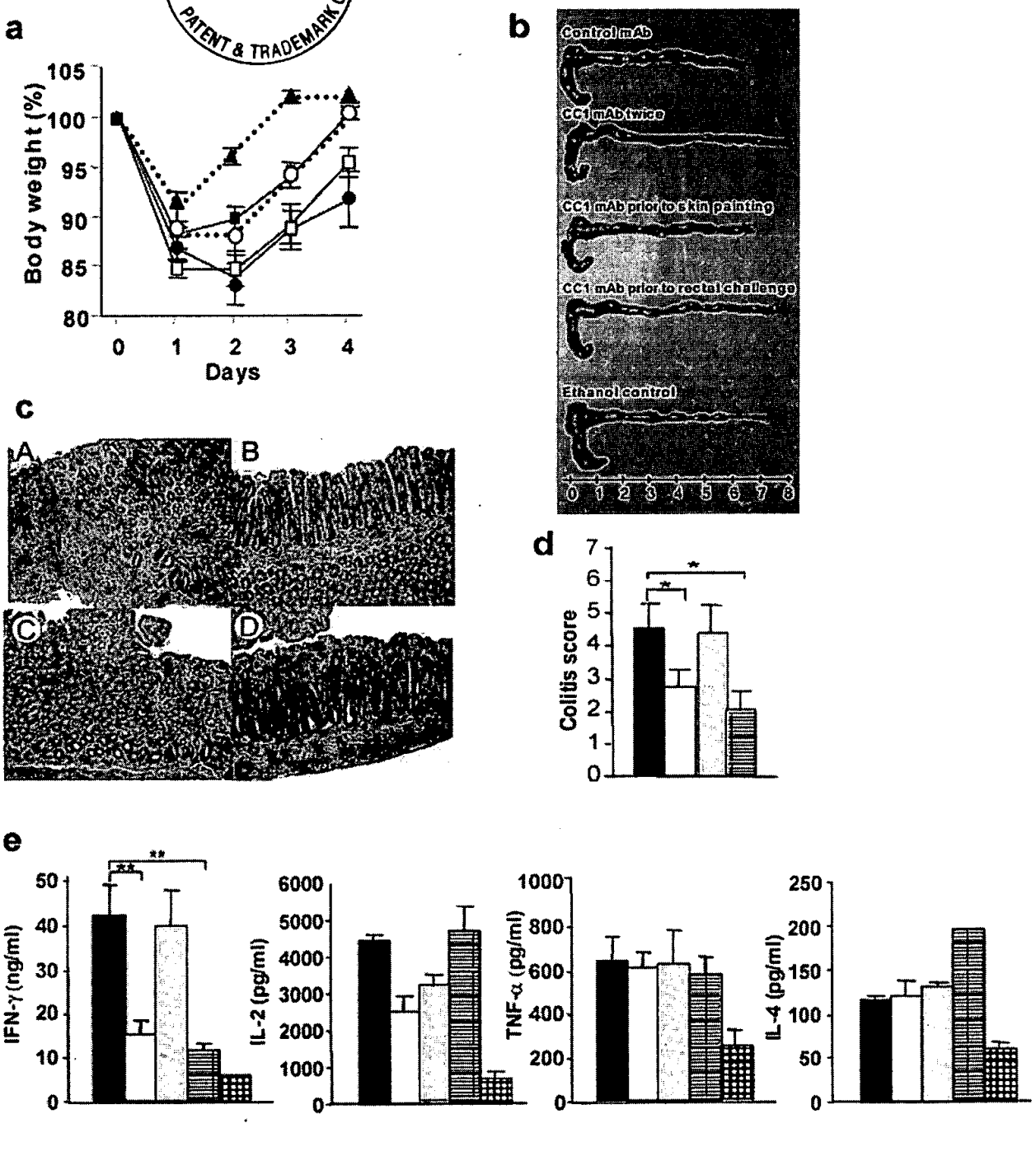
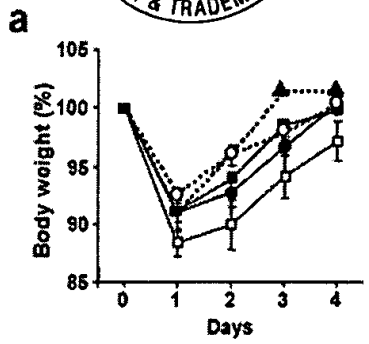
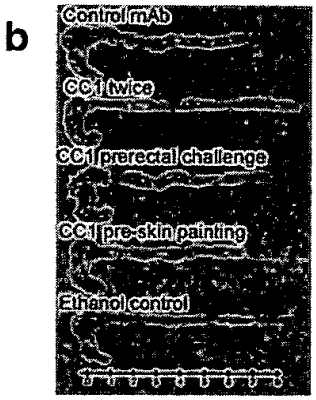
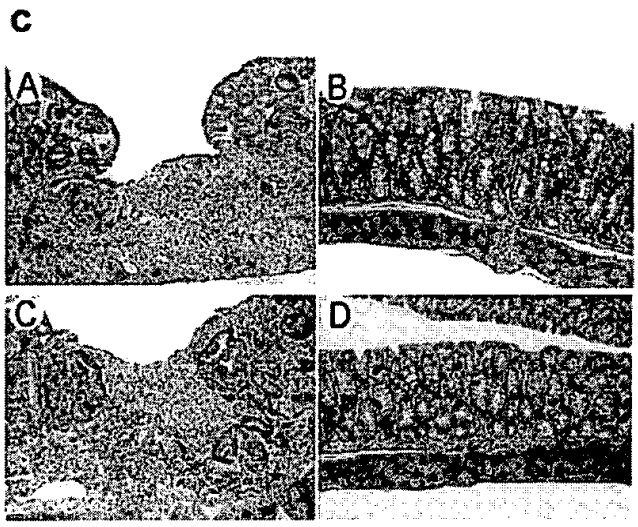


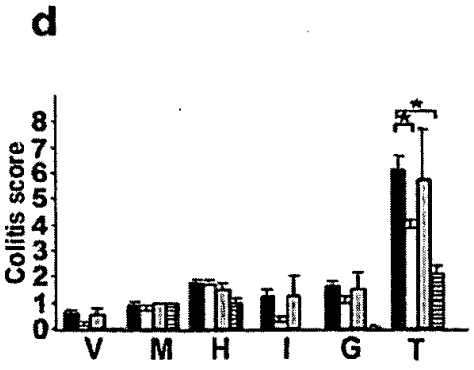
Fig.1

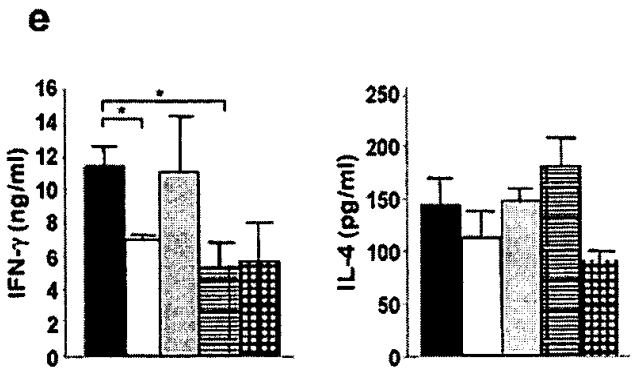
Fig. 2











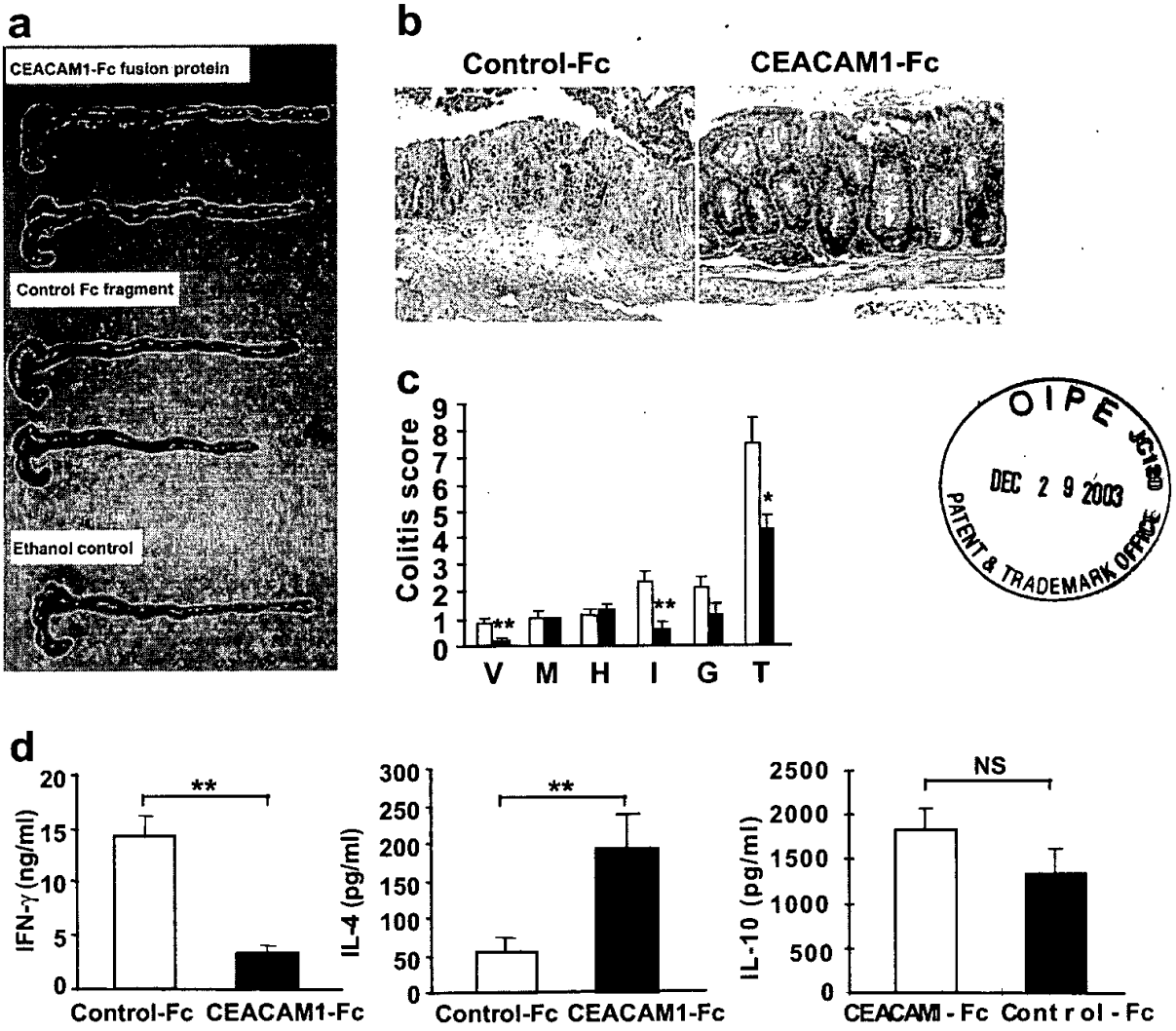


Fig. 5

Figure Legends

Fig. 1 Effect of CC1 mAb injection on the induction of TNBS colitis and cytokine production.

a; Body weight of mice subjected to TNBS colitis treated either with control IgG1 mAb (□), with CC1 mAb prior to skin painting and prior to rectal challenge (twice, ■), prior to skin painting (●) or prior to rectal challenge (○) in C57BL/6 mice are shown. One group was injected with 50% ethanol (▲) instead of TNBS. Data are shown as mean values ± SEM and represent eight mice per group. b; Macroscopic pictures of colons from mice induced TNBS colitis treated with or without CC1 mAb are shown. c; H&E stained pictures from TNBS colitis treated with or without CC1 mAb are shown (magnification; x100). One representative picture from each group of eight is shown (A; control mAb, B; CC1 mAb administered twice, C; CC1 mAb administered prior to skin painting, D; CC1 mAb administered prior to rectal challenge). d; Quantitative histopathologic assessment of TNBS colitis activity shows a significant (*; $P<0.05$ by t-test) suppression in mice treated with CC1 mAb either twice or prior to rectal challenge when compared to the control mAb-treated group. Samples were collected from mice with TNBS colitis treated either with control mAb (■) or CC1 mAb twice (□), prior to skin painting (▣), or prior to rectal challenge (▤). Data are shown as mean values ± SEM and represent eight mice per group. e. Th1 and Th2 cytokine production from LPLs was analyzed by ELISA. Samples were collected from mice with TNBS colitis treated either with control mAb (■) or CC1 mAb twice (□), prior to skin painting (▣), or prior to rectal challenge (▤). One group of mice was administered ethanol without TNBS for the skin sensitization and rectal challenge (▦). CC1 mAb-treated group treated either twice or prior to rectal challenge exhibited significant

suppression of IFN-γ production when compared to the control mAb-treated group (**; $P<0.01$). Data are shown as mean values ± SEM and represent pooled values from eight independent experiments.

Fig. 2 Effect of CC1 mAb injection on the induction of oxazolone colitis and cytokine production. a; Oxazolone colitis was induced in C57BL/6 mice and mice monitored for body weight following various combinations of treatments with CC1 or control mAb. Mice were treated either with control IgG1 mAb (□), with CC1 mAb twice (■), prior to skin painting (●) or prior to rectal challenge (○). One group was subjected to intrarectal administration with 50% ethanol (▲) instead of oxazolone. Data are shown as mean values ± SEM and represent eight mice per group. b; Macroscopic pictures of oxazolone colitis treated with or without CC1 mAb. c; Histologic H&E stained pictures from oxazolone colitis treated with or without CC1 mAb are shown (magnification; x100). One representative picture from each group of eight is shown (A; control mAb, B; CC1 mAb administered twice, C; CC1 mAb administered prior to skin painting, D; CC1 mAb administered prior to rectal challenge). d; Quantitative histopathologic assessment of oxazolone colitis activity shows a significant suppression in mice treated with CC1 mAb either twice or prior to rectal challenge in comparison to the control mAb-treated group (*; $P<0.01$). Samples were collected from mice with oxazolone colitis that were treated either with control mAb (■) or CC1 mAb either twice (□), prior to skin painting (□), or prior to rectal challenge (▤). Data are provided as mean values ± SEM and represent eight mice per group. e; Th1 and Th2 cytokine production from LPLs was analyzed by ELISA. Samples were collected from mice with oxazolone colitis that were treated either with control mAb (■) or CC1 mAb either twice (□), prior to skin painting (□), or prior to rectal challenge (▤). One group of mice

was administered ethanol instead of oxazolone (▣). Mice treated with CC1 mAb either twice or prior to rectal challenge exhibited significant suppression of IFN-γ production when compared to the control mAb-treated group (**; $P<0.01$). Data shown represent pooled values from eight independent experiments. Data are shown as mean values ± SEM.

Fig. 5 Effect of CEACAM1-Fc chimeric protein on the induction of oxazolone colitis.

Macroscopic (a) and histologic pictures (b, original magnification; x100) of colon isolated from mice induced to develop oxazolone colitis treated with CEACAM1-Fc or a control Fc fragment. (c) Colitis scores of colon induced oxazolone colitis treated with CEACAM1-Fc (■) or a control-Fc fragment (□). Mice treated with CEACAM1-Fc exhibited significant reduction in colitis scores (*; $P<0.01$). (d) Th1 and Th2 cytokine production from LPLs was analyzed by ELISA of mice with oxazolone colitis treated either with CEACAM1-Fc (■) or control Fc fragment (□). Suppression of IFN-γ was significant (**; $P<0.01$). Data are shown as mean values ± SEM from four independent experiments.